High-speed acquisition of multi-wavelength fluorescence images: an interview with Jeremy Graham, Cairn Research

Interview conducted by April Cashin-Garbutt, MA (Cantab)

Please can you give an overview of the recent advances in Electron Multiplying Charge Coupled Device (EMCCD) and Complementary Metal Oxide Silicon (CMOS) camera technologies?

On-chip Electron Multiplication is now a mature technology, which has allowed manufacturers to build cameras that are both fast and sensitive; ideal for live cell fluorescence imaging. The central concept is that the photon signal from each pixel is amplified greatly prior to digitization, thus reducing read noise (analogous to a photomultiplier or image intensifier, but with higher inherent sensitivity (Quantum Efficiency)).

Recently there have been useful speed improvements, both in respect to the clock speed of the full sensor and the ability to read sub-regions more rapidly by optically masking the required field of view and using the "dark" pixels for temporary storage. At Cairn we have addressed this by designing suitable mechanical masks – our 'OptoMask' product range.

Scientific CMOS cameras on the other hand, fundamentally operate at MUCH faster clock speeds with many more and smaller pixels and the challenge has been to get the noise and sensitivity comparable, or better than CCD. Modern chip design, with on-board processing, correlated double sampling (CDS) and advanced microlensing has made this a reality. However, when using these cameras an important consideration is that they can generate data at up to 1Gb per second, requiring serious computing power both to capture the data to RAM or hard disk and for subsequent processing. The large sensor size also pushes the field of view limits of today's research microscopes; care needs to be taken to achieve flat field illumination.

What impact have these advances had on the time resolution of affordable wide-field fluorescence microscopes?

Inexpensive scientific CMOS cameras have largely replaced interline CCD cameras for dynamic live-cell imaging.

A fast high-end interline CCD camera can acquire images of around one million pixels at 10 to 15 full frames per second. Scientific CMOS cameras have equivalent pixel size, but much larger sensors and can acquire images of more than four megapixels at 100 frames per second. The speed improvement for a given field of view is greater than an order of magnitude!

EMCCDs are also now offering high temporal resolution, with frame rates of over 60 frames/sec at 512x512 pixels and over 500 frames/sec at 128x128 pixels.

There are many important differences between the two technologies involving rolling shutters, multiplication noise, microlenses, digitisation, FPGA processing, Niquist resolution, and well-depth amongst others. The ultimate decision between EMCCD and scientific CMOS for a given application is fascinating and now depends rather directly on the number of photons that the sample can withstand without damage. Scientific CMOS is better for most applications, but there is still a significant sub-set of low-light and high-speed applications where the EMCCD is preferred. In practise, this subset seems to be rather larger than simple noise calculations would suggest; my suspicion is that the benefits of a high native fill factor and low-noise amplification tend to be overlooked. Please contact us if you want advice for your specific application, we work with all the main companies and strive to be impartial.

What problems can occur when trying to realise these benefits when using multiple dyes or genetic markers?

Biologists often want to use indicators of different wavelength in the same experiment. They may be using several markers to look at different features of their cells, ratio imaging intracellular calcium, or studying co-localisation using FRET. Living samples move, in many cases rapidly, so for accurate overlay the different channels should be acquired at the same time. The traditional method of multicolour fluorescence imaging has been to take sequential images with the illumination and detection light changed mechanically between frames using stepping filter wheels or revolving carousels. These devices have significant inertia and are inherently slow; fine for fixed slides, but not good for live cells.

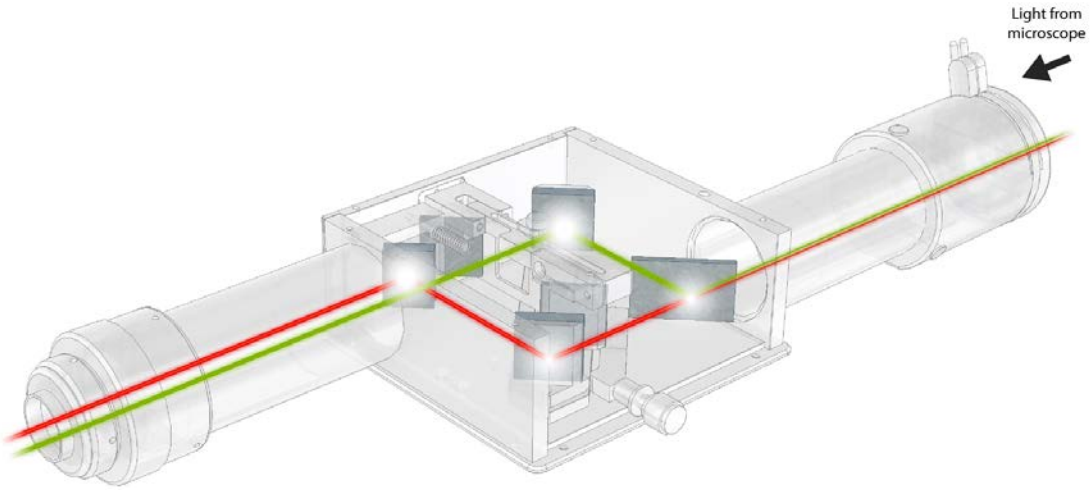
For example, to perform two-colour imaging on a fast camera, you are practically limited to ~5 to 10 frames per second, even though the camera is capable of achieving TEN times better than this. Mechanical delays and the requirement for (serial) software timing have negated much of the benefit of the improvements in camera technology!

How can these problems be overcome?

On the **illumination** side, the timing delays can be overcome using solid-state LED and laser light sources that can be electronically modulated in real time with no mechanical movement. Many research cameras will generate the necessary synchronisation signals, or companies such as Cairn can help out with dedicated timing boxes.

Multi-wavelength **detection**, for example to look at green and red fluorescent proteins with good time resolution, is more difficult. One useful approach is to use multiple cameras or to use multiple regions of the same camera differentiated with spectral beamsplitters to detect different wavelengths simultaneously.

The Cairn MultiCam allows up to four cameras to image the same field of view simultaneously at different wavelengths.



The Cairn OptoSplit II allows simultaneous dual-channel imaging on the same camera sensor with half the field of view.

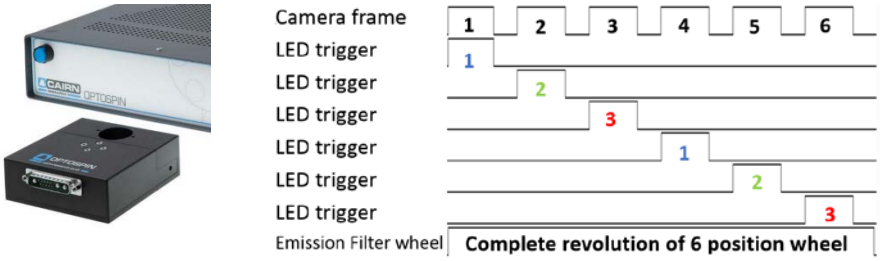
These are both very powerful solutions being truly simultaneous and having no moving parts. However, you either sacrifice the field of view by splitting a smaller region of interest across a single camera sensor, or add cost and complexity by running more than one camera, especially if you want things to be well synchronized.

A further problem is that the cameras / regions are exposed to light at all times, so there is the potential for crosstalk between illumination channels. This is an important consideration and will usually result in images with higher background than if only one illumination and emission wavelength were used at a time.

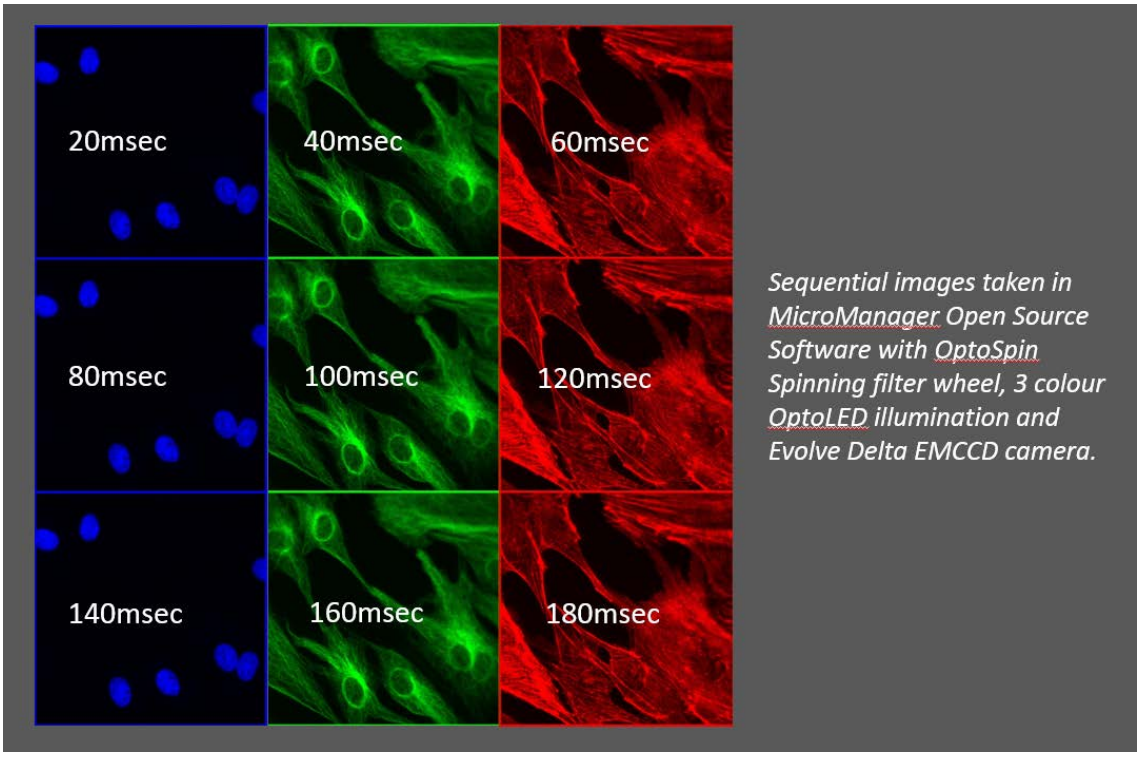
How could a continuously spinning filter wheel be used to help?

By synchronising a continuously spinning emission filter wheel with multi-colour modulatable light sources, it is possible to operate EMCCD or scientific CMOS cameras at very high frame rates whilst taking sequential multi-channel images. Spinning, rather than stepping, avoids the need for repeated rapid acceleration and deceleration cycles and the consequent delays and mechanical vibration.

By synchronising both the camera and lightsource to the spinning wheel it is possible to perform fast, robust multi-wavelength imaging without making any demands of the software. The camera will simply produce an interleaved stack which can be demultiplexed off-line in a few seconds.



The Cairn OptoSpin fast stepping and spinning filter wheel generates all the timing signals for fast imaging at up to six different wavelengths (example shows a three colour protocol).



The time resolution of the camera is fully exploited for three colour imaging without the cost of an emission splitter, multiple cameras or commercial software.

How can crosstalk be removed in simultaneous multichannel imaging?

Although fast, the images shown above are still sequential, so how can we go faster still? If measurements need to be truly simultaneous, then the light **does** need to be separated with spectral beam splitters (dichroic mirrors). The problem, as discussed earlier, is that inevitably absorption light for one fluorophore will interfere with the emission wavelength of a different fluorophore.

For example, we have a sample that is expressed with green and red fluorescent proteins, requiring blue and yellow excitation light respectively.

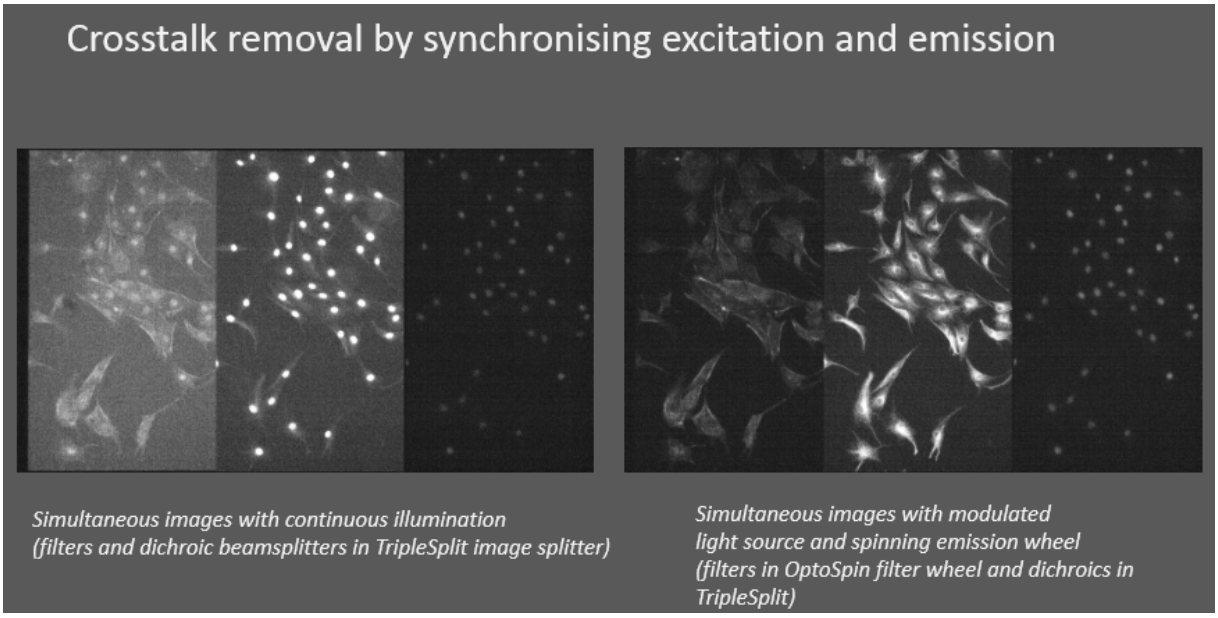
When the green protein is excited with blue light, it will not only emit green fluorescence, but also some component of the same structure will bleed-through into the red channel. Now, imagine if the fluorescence of the green protein is much stronger than the red, then the crosstalk into the red channel from GFP could mask the much lower (desired) signal from the red fluorophore (RFP).

The only way to avoid this is to ensure that when the blue light is on, photons only reach the green detector area and that when the yellow light is on, photons only reach the red detector area, so there's no cross-contamination between the two.

That's very straight forward using conventional “slow” methods, because you would step in the appropriate illumination and detection filters between each measurement. The problem is if two or more emission filters are continuously in the light path, then you can’t avoid this contamination.

Our fix is to synchronise a continuously spinning filter wheel with the camera so that different emission channels are in the light path sequentially within a single exposure.

If the excitation light sources are modulated in sync with the emission wheel, then we can produce frame-rate images at different wavelengths with no crosstalk. This is analogous to strobe photography where the exposure is determined by the illumination and not by the camera integration. The same trick holds for different splitter configurations or multi-camera set ups.



The left hand image shows significant crosstalk which is not evident on the right.

The take home message is that if you want to enjoy the benefits of modern high-speed cameras for multi-channel imaging then there are solutions, they are not expensive and do not require 'fancy' software.

What do you think the future holds for fluorescence imaging?

I think that fluorescence imaging will continue to evolve as a powerful tool for biological research and biomedical analysis. Cheaper and more portable devices are likely to become available for routine medical assays at the point of use, particularly in developing economies.

For high-end imaging one important recent area of improvement relates to the size of the smallest structures that can be accurately measured. This resolution limit for light microscopy is solely a function of wavelength and the Numerical Aperture of the objective, with visible light this works out to around 200 nanometers. Unfortunately, some biologists wish to image very small structures and require better resolution. Until fairly recently this required an Electron Microscopy, which is not friendly for live samples.

Fortunately, a number of biophysicists have been working on this problem for some time and in 2013, three groups were jointly awarded the Nobel Prize for Chemistry for pioneering three different methodologies (tricks) to overcome the diffraction limit, and resolve structures down to tens of nanometers.

The next challenge is to bring these, so called, Super Resolution techniques further into the live cell domain, by improving both the temporal resolution and by making the instruments and sample preparations simpler and therefore accessible to more groups. The experiments now being conducted by the most advanced and best funded researchers are already very impressive.

Another area which is likely to progress in the next few years is that routine fluorescence measurements will be better spectrally resolved, both with more fluorophores being used simultaneously and also with better differentiation of the signal of interest from general background fluorescence.

Cameras are already available with larger sensors than can be used with commercial microscopes, so there are possibilities to use the additional pixels to extract more information, for example real-time simultaneous spatial and spectral imaging.

Although fluorescence measurements are relatively non-invasive compared with alternative techniques, there has recently been a welcome emphasis on reducing the illumination dosages as far as possible to protect both the fluorophores and the biological preparations from photo damage. Also to, hopefully, ensure that the behaviour of the tissue or cells is physiologically relevant and not just a reaction to being bombarded with photons!

Techniques such as Single Plane Illumination Microscopy (SPIM), which is inherently less photo damaging, are likely to become more and more prevalent. Here, instead of the illumination light coming through the objective, it actually comes from the side of the sample as a single plane. The advantage of this is that you are only illuminating the focal plane of the camera, not everything else. By translating the sample through the focal plane, a 3D image can be constructed with significantly reduced photon exposure.

What are Cairn Research's plans for the future?

Our plan is to continue to exploit new, fun, technology to develop (hopefully) innovative products and solutions for fluorescence microscopy and macroscopy.

Microscope illumination is a very competitive area, but we have several niche products in development using dedicated LEDs for fast calcium measurements and optogenetics activation. Laser diodes are getting

cheaper, more reliable and more powerful all the time and we are collaborating with 89 North (www.89north.com) on a very exciting illuminator which should set the new gold standard for many fluorescence and related applications which require intense illumination from a small (point) source.

On the detection side we already have a wide range of accessories encompassing the image splitters, multi-camera adapters and filter wheel mentioned above. We plan to extend this range for simultaneous multi-focal depth imaging and to make use of new (larger) cameras as they become available. We are also collaborating on a novel implementation of camera technology which allows more detailed "interrogation" of the detected signal. This will hopefully prove to be of benefit in Super Resolution microscopy and particle tracking.

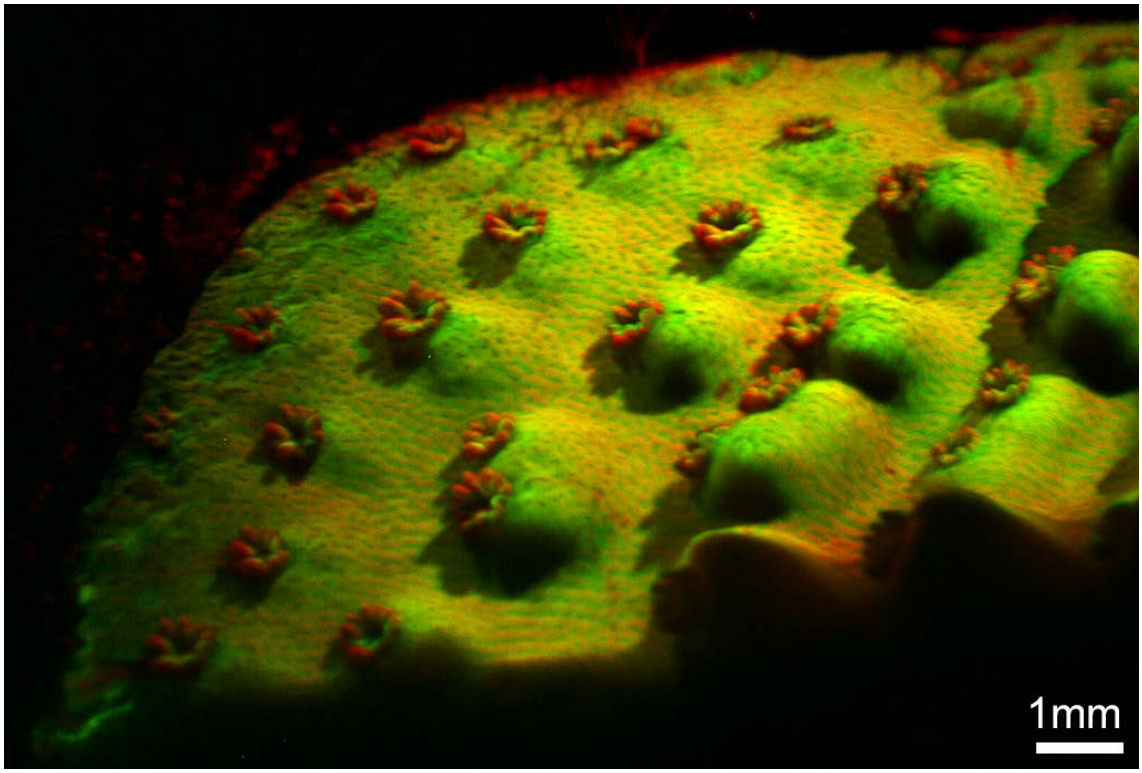
As well as designing and building off-the-shelf products we like to offer turnkey integrated imaging systems and custom designed solutions to answer novel questions which cannot be fully addressed with existing instruments. Historically, many of our bespoke custom designs have evolved into products in their own right and become part of our turnkey product or integrated system ranges. Long may this continue!

We are now looking at making higher-end imaging systems affordable to more groups; putting together systems and sub-systems making use of new technology, but at a price point where individual groups can use it rather than requiring core facilities.

To this end we are developing a stand-alone TIRF / Super Resolution illuminator that is independent of the software and hardware platform and can work with inexpensive, incoherent, light sources.

Finally, we are very keen to do our bit to help make fluorescence experiments less phototoxic and more biologically relevant, and are addressing this issue, by developing new instruments in an attempt to change the way in which experiments are conducted.

As a starting point we feel that there is a need for a simple affordable Single Plane (light sheet) Illuminator.; commercial systems are inflexible and expensive, so many groups are now having to build their own. Self-build may be fun, but it is time consuming and is limiting the uptake of a very useful and important technique. We hope that our (soon to be released), light sheet illuminator will help to address this; results with the early prototypes have been extremely promising.



The growing edge of a reef-building coral, imaged using a Cairn prototype low-cost lightsheet fluorescence microscope. Live imaging helps us understand how climate change affects the growth of these beautiful organisms. However, since they are easily damaged by high light intensities, a special low-light approach using pulsed lightsheets is required.

As a self-financed company with no external shareholders, independence is a key theme in our strategy and philosophy. This allows us to work in very close partnership with researchers and with other 'techie' companies; we greatly value these interactions so please don't hesitate to contact us with ideas or requirements – our ears and eyes are always open!

Where can readers find more information?

They can visit our website or contact us.

- http://www.cairn-research.co.uk/
- http://www.cairn-research.co.uk/contact-us

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About Jeremy Graham

With over twenty years of experience in live-cell microscopy, Jeremy Graham has worked in various technical and sales roles within Cairn Research, culminating in being appointed Managing Director in 2004.

During his time as Managing Director he has helped the business to quadruple in size expanding into many new areas of fluorescence microscopy and related imaging.

He has played a key role in the development of the majority of the products and systems in the Cairn portfolio and has enjoyed successful collaborations with leading researchers in many of the world's most prestigious research institutes.

He regularly contributes to international teaching courses, has been a consultant on several research panels and has contributed to the scientific literature.

Jeremy's role is largely concerned with identifying emerging fields of scientific interest and helping to connect these with new technology to develop novel products and solutions.